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# Basic protocol for transepithelial nasal potential difference measurements

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**Abstract**

Transepithelial nasal potential difference (NPD) measurements assess ion conductance in the upper respiratory epithelium. NPD is useful in assisting in the diagnosis of classical and atypical cystic fibrosis (CF) and of cystic fibrosis transmembrane regulator (CFTR)-related disorders, as well as for monitoring the effect of pharmacological agents and gene transfer approaches to correct the abnormalities of ion transport in CF. The article summarizes the objectives and the principle of NPD measurements, describes a hands-on protocol of the procedure and provides quality control measures, practical hints and troubleshooting.


*Keywords:* Basic defect; Cystic fibrosis; Diagnosis; Nasal potential difference

## 1. Objectives

Cystic fibrosis (CF) is characterized by abnormal airway epithelial sodium ($Na^+$) and chloride ($Cl^-$) transport that reflects defects in the cystic fibrosis transmembrane regulator (CFTR) gene. Measurements of transepithelial potential difference (PD) have been performed in the nose (and lower airways) of human subjects [1], and patients with CF have a characteristic pattern of bioelectric properties which reflect accelerated $Na^+$ transport (absorption), and (absent) or reduced CFTR-mediated $Cl^-$ secretion [1–4]. These bioelectric indices have been validated by extensive in vitro studies of freshly excised human tissues, as well as in vitro studies of airway epithelial cells in culture, including intracellular ion-selective microelectrode impalements, as well as characterization of the multiple components of the transepithelial barrier, i.e., the apical and basolateral cell membranes and the paracellular (shunt) pathway. Because in vivo measurement of nasal potential difference (NPD) can define ion transport abnormalities that are characteristic of CF, this technique may be useful in assisting in the diagnosis of cystic fibrosis, as well as for monitoring the effect of pharmacological agents and gene transfer approaches to correct the abnormalities of ion transport [3–6].

*Abbreviations:* AT; anterior turbinate; IT, inferior turbinate; NPD, nasal potential difference; PD, potential difference; PE, polyethylene.

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## 2. Principle of the assay

Active ion transport by the respiratory epithelium is thought to play an important role in the regulation of the volume and composition of airway surface liquid, and ultimately contributes to the efficiency of mucociliary and cough clearance. In vitro, freshly excised airway epithelial tissues can be studied in Ussing chambers, and when the transepithelial electric PD is clamped to zero (''short-circuited''), basal patterns of ion transport can be directly determined by isotopic measurements of the transepithelial flux of specific electrolytes, including $Na^+$ and $Cl^-$ ions. Thus, there are defined correlates of ion flux and bioelectric parameters of active ion transport and tissue (barrier) resistance. Because the PD generated by the airway epithelium reflects the relationship between the magnitude and direction of the net transepithelial active ion transport and the epithelial ion conductance, the transepithelial PD (voltage) serves as an index of ion transport and ionic conductances (permeability).

The net flux of ions cannot be measured in vivo, but the interpretation of bioelectric changes can be inferred from principles developed from studies of human tissues in vitro, and from basic principles of ion transport and membrane physiology. The general principle for recording transepithelial PD is that the subcutaneous space is isoelectric at all locations in the human body; thus, the transepithelial voltage can be quantified in magnitude and polarity, by referencing the PD to 'zero' in submucosal space. In brief, a subcutaneous reference electrode located anywhere in the body would be the equivalent to a reference electrode located in the submucosal space of the nasal epithelium [4].

The voltage between the reference electrode and the exploring electrode accurately reflects the transepithelial PD, assuming the system is appropriately established. The simplest way for this to be demonstrated is that abrasion of the epithelium reduces the transepithelial potential to zero, which demonstrates that the development of transepithelial PD requires an intact epithelial surface, and the subcutaneous reference electrode (in the forearm) is isoelectric with submucosal space in the nasal cavity. The polarity and magnitude of the voltage is also in the range of values recorded in vitro in multiple human cell systems. Finally, the local application of drugs also provides clues to the nature of the active ion transport process across the epithelium [4,5].

Specifically, drugs with relatively specific actions, such as the $Na^+$ conductance inhibitor amiloride, inhibits $Na^+$ entry across luminal membrane of the nasal epithelial cells in a dose-dependent fashion, which results in the reduction of transepithelial difference in vivo. Similarly, the measurement of $Cl^-$ conductance (''secretion'') can be assessed by establishing a gradient for $Cl^-$ ion to exit the luminal (apical) membrane of the airway cell by perfusing the surface of the epithelium with a $Cl^-$-free solution, whereby $Cl^-$ ions are replaced by an impermeant anion, such as gluconate. Pharmacological activation of the $Cl^-$ channel may be tested by prior establishment of an electrogenic gradient for $Cl^-$ to exit the cell (and thereby increase, i.e., hyperpolarize, the transepithelial PD). Although the role of the paracellular shunt cannot be well defined in a quantitative sense in vivo, the magnitude of the apical membrane ion selectivity far exceeds any selectivity of the shunt, and therefore the latter plays a minor role in the overall value of the transepithelial PD.

## 3. Basic protocol [4,7,8]

NPD is measured between a fluid-filled exploring bridge positioned on the airway surface and a fluid-filled reference bridge in the subcutaneous space. This approach also ensured application of drugs directly at the locus of PD measurement ('Superfusion' method). The regimen uses balanced Ag/AgCl electrodes (or saturated calomel half-cells) connected to a high-impedance voltmeter, an exploring bridge (double lumen catheter) and a subcutaneous reference electrode (22- to 24-gauge (ga.) needle) that are both perfused with isotonic Ringer's solution. The tip of the exploring tubing is placed onto respiratory mucosa under the concha nasalis inferior. To study the response of PD to several drugs, the superfusion solutions are applied via the exploring electrode connected by a three-way valve or through the second lumen of a double-barreled catheter.

### *3.1. Preconditions*

Compliance: NPD with superfusion is possible in infants and in children older than 5 years with intact nasal mucosa (without, e.g., acute rhinitis).

## 4. Materials: superfusion solutions

Solution A: Custom Ringer's. 135 mM NaCl; 1.2 mM $MgCl_2$; 2.25 mM $CaCl_2$; 2.4 mM $K_2HPO_4$; 0.4 mM $KH_2PO_4$. Solution B: 0.1 mM amiloride hydrochloride (HCl) in Ringer's solution. Solution C: Zero $Cl^-$ solution (+amiloride): 135 mM sodium gluconate, 1.2 mM $MgSO_4$, 2.2 mM Calcium gluconate, 2.4 mM $K_2HPO_4$, 0.4 mM $KH_2PO_4$, 0.1 mM amiloride HCl. Solution D: 0.01 mM isoprotenerol HCl in solution C (caution: vials of isoprotenerol contain $Cl^-$). Solution E: 0.1 mM ATP in solution D. Check that pH is 7.4, range 7.0–7.6. Solutions A, B, and C may be refrigerated for up to 3 months or frozen for up to 6 months, solutions D and E are freshly prepared within 2 h prior to use. Prior to use, all solutions must be filtered with a 0.22-μm filter.

## 5. Equipment

### *5.1. Exploring electrode*

The exploring electrode is an isotonic NaCl/Ringer's saline-perfused exploring bridge positioned on the airway

surface. Exploring bridges are prepared either by filling lengths of polyethylene (PE) tubing (PE-50 to PE-160) with 3 M KCl in 4% agar or by a fluid-filled double-lumen catheter continuously perfused with warmed (24–37 °C) gassed isotonic NaCl/Ringer's saline (0.2–0.4 ml/min). Contact with the nasal surface is ensured by perfusion. The exploring bridge can consist of a vinyl catheter (example: umbilical vessel catheter, 5Ch 1.7mm). Most catheters can be used repeatedly following gas sterilisation.

### 5.2. Reference electrode

The reference electrode is an isotonic NaCl or Ringer's saline-perfused bridge in the subcutaneous space of the lower arm. The reference bridge is a 19- to 24-ga. needle that contains isotonic Ringer.

### 5.3. Measuring electrode, voltmeter

A high-input impedance ($10^8$–$10^{12}$ Ω), low-resistance voltmeter amplifier is connected to two silver/silver chloride half cells inserted in gas tight Erlenmeyer flasks which are filled with 3 M KCl solution. These Erlenmeyer flasks are connected via 3 M KCl 4% agar gel bridges in gas tight glass tubes with two other Erlenmeyer flasks filled with isotonic NaCl solution. From those flasks, both the subcutaneous reference electrode and the exploring catheter are connected via isotonic NaCl 4% agar gel bridges in normal infusion tubings. This described regimen of agar gel bridges avoids offset potentials and exhibits stable and reproducible potential for several years. Alternatively, Ag/AgCl electrodes connected by a Luer lock port with the flow-through system are possible. Other groups use calomel half-cells connected via a Ringer–4% agar bridge to a high-impedance voltmeter.

### 5.4. Data recording

Data are recorded on a chart recorder or a computerized recording system. Indicate the trace zero, voltage span, and chart speeds. The offset voltage should initially set to zero. If the final offset voltage at the termination of the procedure differs from the initial offset by more than 5 mV, designate the trace 'invalid'.

## 6. Measuring the NPD[1]

### 6.1. Protocol

(1) Prepare reagents.

(2) Prior to each procedure, check the set-up and use new tubing circuits to avoid inter-patient contamination. A fresh sterile exploring catheter is used for each measurement.

(3) The perfused solutions should be warmed so that the temperature exiting from the exploring catheter is in the range from 32 to 37 °C (Some laboratories work at ambient room temperature, although it should be noted that a warmed nasal perfusate gives a greater $Cl^-$ conductance [9]).

(4) Check the zero offset. Prior to use, the electrodes are calibrated by placing the reference electrode in close contact with the exploring electrode to test asymmetry before and after examination of each subject. When electrode pairs differ in PD by more than 1 mV, the bridges are discarded.

(5) Check the conductivity. The conductivity of the bridges is assessed by a preliminary measurement of skin PD at the palm of the hand. If a stable PD cannot be recorded (< 3% of cases), the reference bridge is replaced, the perfusion bridge flushed, and the test repeated. Values for baseline PD measurements are accepted if PD measurements are stable for more than 3 s and if the PD measurement at the palm of the hand are comparable at the beginning and end of the study.

(6) Position of the subject. The subject should sit relaxed and comfortably in an armchair so that the nasal perfusate flushes from the nostril without any discomfort.

(7) Insert the exploration catheter into the nostril using an endoscope or otoscope to visualize the inferior turbinate (IT). Record voltage at the tip of anterior turbinate (AT), advance the catheter through the inferior meatus and record voltage every 0.5–1 cm, using the tip of the AT as landmark. The AT of the IT is a convenient anatomic reference point (squamous epithelium). Values of a measurement are accepted if the PD of the AT at the beginning and the end of the procedure is comparable ($\pm$ 5 mV).

(8) Place the catheter at the site of most negative signal. Fix the exploring catheter with tape on the patient's forehead. The catheter should have enough tension to stay at the IT. Confirm position and depth of tip with endoscope or otoscope.

(9) Start continuous voltage recording.

(10) Start perfusion with solution A. For each solution, NPD is measured for a minimum of 3 min. A steady voltage tracing should be achieved for at least 15 s (<3 mV/min drift). All solutions are perfused at 5 ml/min (there is no consensus about the optimal flow rate that vary by laboratory between 1.5 and 5 ml/min, although low flow rates have been demonstrated to cause substantial variability [10]). Solution changes should be marked in the trace.

(11) Replace solution A by solution B.

(12) Replace solution B by solution C.

(13) Replace solution C by solution D.

(14) Replace solution D by solution E.

(15) Stop perfusion, verify the position of the tip of the catheter, then record AT potential again.

(16) Remove the catheter, replace solution E by solution A and flush with solution A to remove prior reagents.

(17) Repeat steps (7)–(15) in the other nostril.

(18) By the end of the measurement, repeat steps (4) and (5).

[1] Modified NPD protocols are available from T. Leal [11] and M. Hug (email: hugma@web.de).

### 6.2. Evaluation and interpretation of data

There is still substantial variation between laboratories with respect to the absolute PD values, but the differential responses of CF subjects and non-CF controls are clearly discerned. The basal PD in non-CF subjects is typically $-20 \pm 10$ mV. The change in PD with amiloride superperfusion is $11 \pm 6$ mV. The cumulative responses to $Cl^-$-free solution and to isoproterenol sum up to about $-19 \pm 11$ mV. The mean basal PD of CF patients is approximately double the value in controls, $-45 \pm 10$ mV. Amiloride has a larger inhibitory effect in CF patients, $30 \pm 15$ mV. There is no or only a minimal response to $Cl^-$-free solution and isoproterenol in CF patients which can be taken as the most sensitive and specific criterion to make a CF diagnosis. The PD response to ATP in normals ($-5 \pm 3$ mV) is typically increased in CF patients to about $-10 \pm 5$ mV.

## 7. Troubleshooting and practical hints

Variability of NPD measurements can reduce the power of studies using NPD as outcome measures. They may cover the following: the material used (catheters, electrodes); the offset of the electrodes; the positioning and fixation of the catheter; the measure itself (stability of the measure, duration of the experiment); the tested subjects (age, clinical status). The following practical hints may be helpful for in-house validation and standardization of the procedure.

### 7.1. The material

The exploring bridge must be a double lumen catheter with the opening of both lumina at the same site. One lumen is filled with a fluid allowing ionic conductance and connected to a high-impedance voltmeter via the electrode. The second lumen is perfused with the different solutions using a pump that provides a continuous flow throughout the perfusion period.

The types of catheter used vary according to the operators. The most frequently used are: 8-Fr Foley paediatric urinary catheter, 4 Fr double-lumen central venous catheter, hand-held single-lumen PE-50 tubing, umbilical vessel catheter. Some operators modify the Foley catheter by cutting the balloon area and then sticking it to the distal part. At the Necker Enfants Malades hospital, the Ethics Committee pointed out that this procedure alters and desterilizes a material designed for single use. Besides, the distal part can be inhaled. Hence, the cutting of catheters is discouraged.

The walls of the catheter must be rigid enough to wedge securely into the right position. Silicone appears to be a good compromise between the tolerance of the experimentation by the patient and the commodity of use for the operator. The diameter of the catheters is also a source of variability because the catheters with a small diameter tend to slip out of position, which could result in inaccurate measurements. For adults, and children older than 8 years of age, a catheter with external diameter of 2.7 mm may be used. The catheter could be marked at 0.5-cm intervals in order to control the position of fixation. The Hannover group uses an umbilical vessel catheter (5Ch 1.7 mm) that allows correct positioning of the tip in the nostril and fixation by tape on the forehead with minimal discomfort for the subject. A catheter has been developed for the investigation of infants.[2]

The fluid filling the bridge linked to the exploring electrode is either the Ringer's solution in 4% agar [1], or an equal mixture of Ringer's lactate and electrocardiographic cream [3]. In the second case, the two components must be gently mixed and left to set at least 1 h before experimentation, in order to have the least amount of bubbles in the solution.

The reference electrode is generally placed on the inner forearm after abrading the skin. This operation is necessary to create a suitable site of zero potential. A handheld motor unit containing a diamond-tipped dental burr can be used. The reference electrode is taped over this site and diluted (1:1) electrode gel is injected through the hole into the electrode to allow electrical contact [3]. Other operators prefer a reference bridge with a 19- or 21-ga. needle filled with Ringer's 4% agar inserted into the subcutaneous space of the forearm linked to the reference electrode [1,4]. This is an additional apparatus that can frighten children, but has the advantage of having a direct access to the subcutaneous space of the forearm, which is isoelectric with the submucosal space of the airway epithelia.

### 7.2. Offset of the electrodes

Prior to recordings, the offset of the electrodes must be measured and appropriate corrections made to record values. Under short-circuit conditions, 0 mV is obtained when the reference electrode is in contact with the exploring electrode. Values $> \pm 5$ mV are unacceptable and the electrodes must be changed.

The conductivity of the bridge must be assessed by preliminary measurements of skin PD. The PD of the tip is noted. Acceptable values range from $-30$ mV and upwards. If a stable PD is not recorded, the bridges must be changed or the skin more deeply abrased or the subcutaneous skin bridge changed.

### 7.3. Positioning and fixation of the catheter

Proper positioning along the IT is critical to obtain accurate measures. It is a very important moment in the

[2] This is available at Marquat Génie Médical (Boissy Saint Léger, France).

experimentation. If the catheter is not positioned at the right site or not properly fixed, this leads to inaccurate and possibly unstable measurements. The catheter must be introduced into the nostril under visual guidance with an otoscope and a nasal speculum or a plastic autoscope speculum. The anterior tip of the IT provides a convenient anatomic reference point. Initial PD measurements are made at that site. The bridge is then advanced gently through the inferior meatus along the floor of the nose and a PD profile of the turbinate is obtained by recording 5 s measurements at 0.5, 1, 1.5, 2 and 3 cm posterior to the anterior tip. The PD in the anterior tip is low; there is a sharp increase under the turbinate and a subsequent gradual decrease more posteriorly. The catheter is withdrawn slowly from the 3-cm site while readings are noted. A maximal stable ( $\pm$ 1 mV) PD is sought and recorded if the voltage is stable for more than 10 s. The maximal stable PD is usually obtained 3 cm from the anterior tips of the turbinate. It is verified by slow rotation of the catheter that the value is maximal.

The fixation must avoid the slipping out of the catheter because of the leaning forward position of the patients and the continuous perfusion of the fluid which can moister the fixation. Some operators ask the patient to hold the catheter and to maintain this position during the whole procedure. However, this carries the risk of moving the catheter from the initial site of measurement and is difficult to obtain in children. Fixation of the catheter with tape at the forehead is recommended.

### 7.4. NPD measurements

The exploring bridge is perfused with drug-free Ringer's at a perfusion rate as low as 0.2 ml/min for at least 30 s. When a stable PD is recorded, the drug-free Ringer's perfusion is stopped and perfusion of drug is initiated. The perfusion rate throughout the experimentation must be at least 4 ml/min. It has been demonstrated that a slower flow (2.2 versus 5 ml/min) is a source of variability [10]. The effects of the drug must be recorded during at least 180 s for the following reasons. First, the new perfusate reaches the catheter approximately 45 s following solution change because of the 3 ml dead space of the perfusion system. Second, a stable voltage plateau must be achieved. The equilibration time after adding amiloride or isoproterenol is typically about 1 min. In contrast, after the change to the low $Cl^-$ solution NPD is stable only after 4 min. It is therefore mandatory to consider that the last 60 s are taken as the final reading for each solution. Recordings for 3 min after adding amiloride or isoproterenol and for 5 min after changing to low $Cl^-$ solution are recommended. In any case, if a steady state is not obtained after this delay, the recording must be prolonged. Zero offset and conductance should be checked at the beginning and the end of the procedure.

The solutions can be administered at room temperature. However, administration of drug at 37 °C improves the sensitivity of the measurement.

### 7.5. The patients

Acute inflammation of the nasal respiratory epithelium that disrupts the epithelial integrity, localized hemorrhage or viral infection can artificially impair detection of NPD. Therefore, it is recommended to delay the measure for 1 month if there is a history of hay fever, chronic rhinitis or any symptoms of acute rhinitis in the second week prior the study and not to include regular smokers. Age must be considered because PD values are significantly lower in older men [1].

Finally, differences in operator skill and technique are the most important causes of the differences in measurement variability. This can be minimized by careful operator training, standardization of measurement protocols and ongoing monitoring of individual operator performance.

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